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Research article

Delineation of RAID1, the RACK1 interaction domain located within the unique N-terminal region of the cAMP-specific phosphodiesterase, PDE4D5

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Abstract

Background: The cyclic AMP specific phosphodiesterase, PDE4D5 interacts with the β-propeller protein RACK1 to form a signaling scaffold complex in cells. Two-hybrid analysis of truncation and mutant constructs of the unique N-terminal region of the cAMP-specific phosphodiesterase, PDE4D5 were used to define a domain conferring interaction with the signaling scaffold protein, RACK1.

Results: Truncation and mutagenesis approaches showed that the RACK1-interacting domain on PDE4D5 comprised a cluster of residues provided by Asn-22/Pro-23/Trp-24/Asn-26 together with a series of hydrophobic amino acids, namely Leu-29, Val-30, Leu-33, Leu-37 and Leu-38 in a 'Leu-X_{aa}-X_{aa}-X_{aa}-Leu' repeat. This was done by 2-hybrid analyses and then confirmed in biochemical pull down analyses using GST-RACK1 and mutant PDE4D5 forms expressed in COS cells. Mutation of Arg-34, to alanine, in PDE4D5 attenuated its interaction with RACK1 both in 2-hybrid screens and in pull down analyses. A 38-mer peptide, whose sequence reflected residues 12 through 49 of PDE4D5, bound to RACK1 with similar affinity to native PDE4D5 itself (K_a circa 6 nM).

Conclusions: The RACK1 Interaction Domain on PDE4D5, that we here call RAID1, is proposed to form an amphipathic helical structure that we suggest may interact with the C-terminal β-propeller blades of RACK1 in a manner akin to the interaction of the helical G-γ signal transducing protein with the β-propeller protein, G-β.

Background

cAMP is a ubiquitous second messenger that regulates numerous key physiological processes [1–6]. Its levels are determined both by controls on its rate of synthesis through adenylyl cyclase activity and its rate of degradation through cAMP phosphodiesterase (PDE) activity [1]. A large multigene family encodes many proteins that exhibit PDE activity [6–13]. Of these, the PDE4 cAMP-specific phosphodiesterases have attracted considerable attention as inhibitors that are selective for them have behavioral, anti-inflammatory and smooth-muscle relaxant activity in humans [7,8,10,13–15]. PDE4 enzymes can be differentiated from other cyclic nucleotides phosphodiesterase (PDE) families on the basis of sequence differences in their catalytic region and by their ability to be specifically inhibited by the drug, rolipram [10]. They are also characterized by unique regulatory regions located in the amino-terminal half of the proteins, called Upstream Conserved Regions 1 and 2 (UCR1 and UCR2) [7]. 'Long' PDE4 isoforms exhibit both UCR1 and UCR2, whilst 'short' isoforms lack UCR1 and 'super-short' isoforms lack UCR1 and have a truncated UCR2. In long PDE4 isoforms, UCR1 and UCR2 appear to interact with each other [16] in order to form a regulatory module that mediates the functional outcome of phosphorylation by PKA [17,18] and ERK [19]. The mammalian PDE4s comprise a large family of isoforms, encoded by four different genes (*PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*), with additional diversity being generated by alternative mRNA splicing and the use of alternative promoters [10]. A characteristic feature of these different isoforms is their unique N-terminal regions, which are believed to play a key role in the intracellular targeting of these isoforms [1,10,20]. For example, the different N-terminal regions of PDE4A1 [21,22] and PDE4A4/5 [23,24] determine distinct patterns of intracellular targeting. Thus PDE4A1 is an exclusively membrane-associated species that is targeted to the Golgi, whilst PDE4A4/5 is localised to perinuclear and cell cortical regions in COS7 cells.

PDE4D5 is one of five different isoforms encoded by the *PDE4D* gene and is found in a variety of tissues and cell types, including the brain [25]. PDE4D5 can be distinguished from other PDE4D isoforms by the presence of a unique amino-terminal region of 88 amino acids, which is highly conserved among mammals [25]. We have recently demonstrated [26] that this PDE4D isoform can interact with the WD-repeat signalling "scaffold" protein, RACK1 [27–30]. This was done using independent methods to demonstrate that RACK1 and PDE4D5 interact; namely two-hybrid screening, pull-down assays with recombinant RACK1, binding studies with purified recombinant proteins, and also by co-immunoprecipitation of native, endogenously expressed RACK1 and PDE4D5 [26]. Native forms of these two proteins thus interact in a variety of different cell types [26]. RACK1 is a 36 kDa WD-repeat protein that was first identified as a protein that could bind to certain protein kinase C (PKC) isoforms subsequent to their activation by either diacylglycerol or phorbol esters such as PMA [27–30]. However, RACK1 has also been shown to interact with other proteins such as the β-subunit of integrins [31], the common beta-chain of the IL-5/IL-3/GM-CSF receptor [32], the Epstein-Barr virus cytoplasmic A73 protein [33] and SRC family tyrosyl protein kinases [34]. Thus RACK1 appears to act as a scaffold or anchor protein [30] as do other WD-repeat proteins such as Gβ and β '-COP [35–37].

Previously, it has been shown that a small cluster of 4 amino acids in the unique N-terminal region of PDE4D5 is needed for its interaction with RACK1 [26]. Here, using a program of deletion and mutagenesis strategies we show that a much more extensive surface within the unique N-terminal region of PDE4D5 is needed, involving a series of hydrophobic amino acids extending from Leu-29 to Leu-38. We call this the RACK1 Interaction Domain, RAID1 and suggest that it forms a helical region able to interact with RACK1.

Results

Long PDE4 isoforms are characterised by the presence of two blocks of sequence, called UCR1 and UCR2, located between the conserved catalytic unit and the extreme N-terminal region that is unique to each isoform (Fig. 1) [10,38]. The unique amino-terminal region of PDE4D5 (Fig. 1) consists of a block of 88 amino acids that has no homology with any of the other four PDE4D isoforms [25]. We have demonstrated previously [26] that this region of PDE4D5 is necessary and sufficient for it to bind to RACK1, thus explaining why PDE4D5 uniquely amongst the PDE4D isoforms is able to interact with RACK1. Within the PDE4D5 N-terminal region we discovered [26] that the Asn-22/Pro-23/Trp-24/Asn-26 grouping of amino acids (Fig. 1) was crucial in allowing PDE4D5 to bind to RACK1. The discovery of this essential group of amino acids was achieved through a strategy of progressive N-terminal deletions and subsequent point mutations within the 88 residue N-terminal region of PDE4D5 [26].

Mapping of the site for RACK1 interaction within the unique N-terminal region of PDE4D5

Using an ELISA assay, we confirmed previous observations [39], that PDE4D5 interacted with RACK1 with a K_a of 4 – 12 nM (range; n = 5 separate experiments). In contrast to this, using both ELISA and TNT competition assays, we failed to observe any binding to RACK1 of a 5-mer peptide (sequence = NPWLN) that contained the Asn-22/Pro-23/Trp-24/Asn-26 grouping (Fig. 1), which we had previously identified as being pivotal for interaction

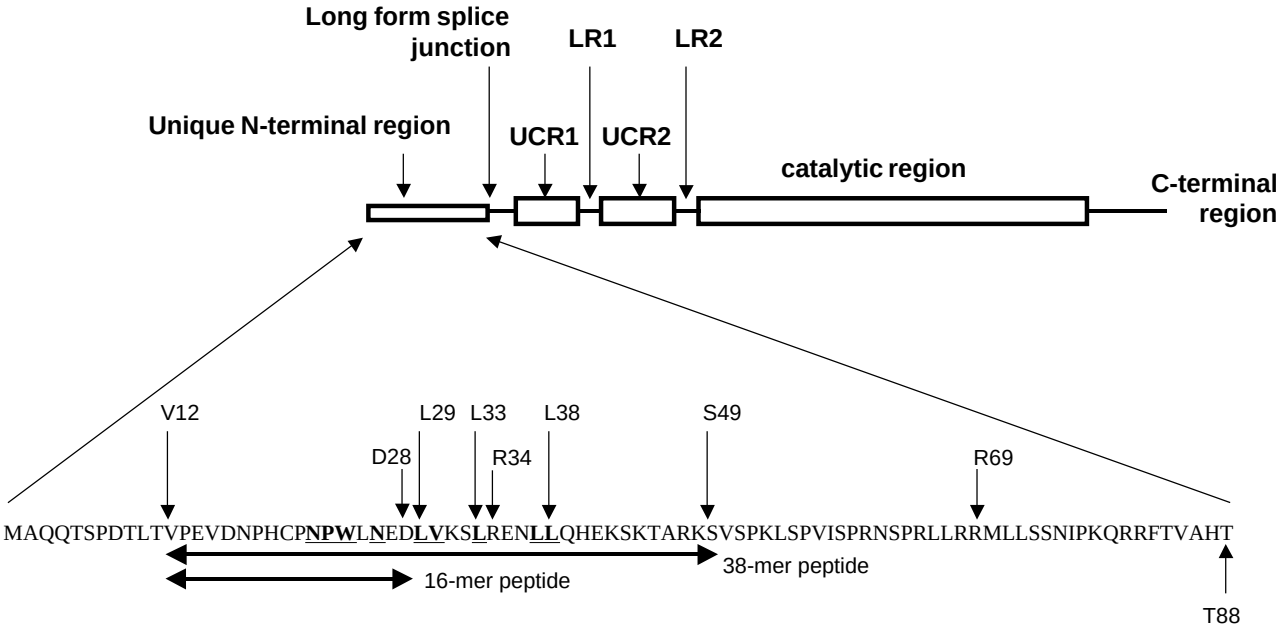

Figure 1
The unique N-terminal region of PDE4D5 This shows schematically the domain structure of the PDE4D5 long isoform together with the amino acid sequence (GenBank™ accession number AF012073) of its unique 88 residue N-terminal region. Indicated by a horizontal arrow is the sequence of both the 16-mer and 38-mer peptides that were assessed for binding to RACK1. In bold typeface are shown the residues of the essential Asn-22/Pro-23/Trp-24/Asn-26 grouping and also those that form the essential hydrophobic ridge, Leu-29, Val-30, Leu-33, Leu-37 and Leu-38. Also indicated are the positions of the various truncated species made with associated residue numbers.

of PDE4D5 with RACK1. Indeed, we were further surprised to discover that a 16-mer peptide (PEVDNPHCPNPWLNED), representing residues 13 to 28 of PDE4D5 and thus also containing the Asn-22/Pro-23/Trp-24/Asn-26 grouping (Fig. 1), showed little if any interaction with RACK1 (K_a > 1 μM; n = 5).

These data suggested to us that amino acids in addition to those within the Asn-22/Pro-23/Trp-24/Asn-26 grouping might be involved in allowing the efficient binding of PDE4D5 to RACK1. We thus set out to try and identify such determinants. The discovery of the Asn-22/Pro-23/Trp-24/Asn-26 grouping was based upon a strategy of progressive N-terminal deletions of the 88 residue N-terminal region of PDE4D5, followed by scanning mutagenesis of amino acids Pro-21 through Glu-27. Thus any additional RACK1-interacting residues must be C-terminal to the Asn-22/Pro-23/Trp-24/Asn-26 grouping. To evaluate this we instigated a strategy of progressive C-terminal truncation of the unique N-terminal region of PDE4D5 in a 2-hybrid screen. We have previously confirmed [26] the fidelity of such an approach by using a variety of independent biochemical analyses, including co-immunoprecipitation, pull down assays with GST-RACK1 and the binding of purified components. The full N-terminal region of PDE4D5 interacted with RACK1, as did the 12–88 N-terminal truncate in this 2-hybrid analysis (Fig. 2). However, the C-terminally truncated construct, encompassing residues 12 through 28 of PDE4D5, failed to interact with RACK1 (Fig. 2), despite the fact that such a region contained the Asn-22/Pro-23/Trp-24/Asn-26 grouping. Such a result indicates strongly that additional residues, located C-terminal to the Asn-22/Pro-23/Trp-24/Asn-26 grouping, are required for PDE4D5 to bind to RACK1. Further truncation analyses demonstrated that a C-terminal truncate, containing residues 12 through 49 of PDE4D5, was able to bind to RACK1 (Fig. 2). This suggests that certain amino acids, located within positions 27 through 49 in the sequence of PDE4D5, are also required for RACK1 interaction.

Previously we have shown [26] that Glu-27 was not involved in RACK1 interaction. Here then we set out to determine which specific amino acids in the 28 through 49 region of PDE4D5 are necessary for the interaction with RACK1. Again, a 2-hybrid assay was used to test for the ef-

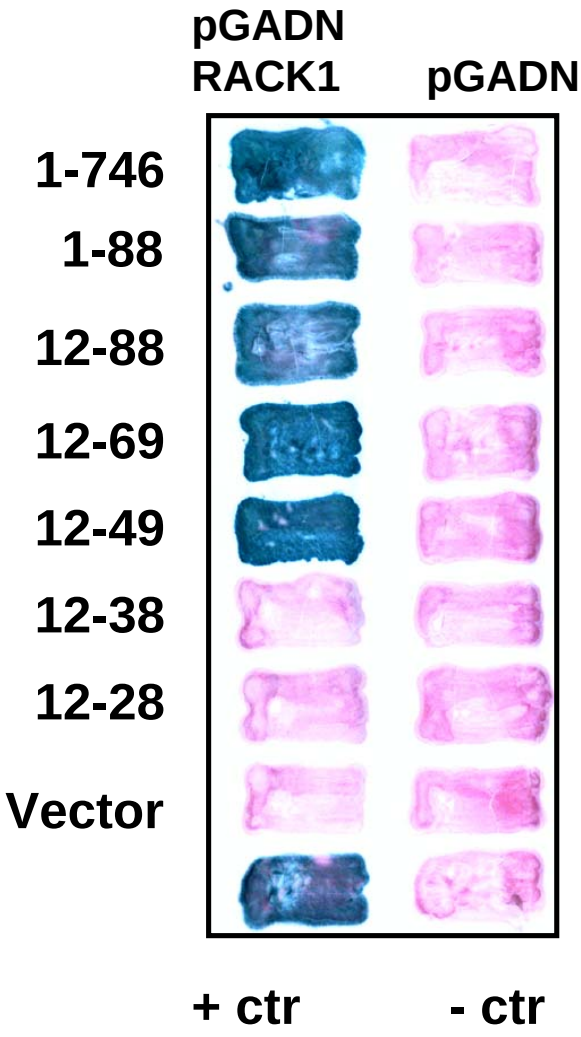

Figure 2
Truncation studies within the unique N-terminus of PDE4D5 used to probe its interaction with RACK1 using a 2-hybrid screen. Plasmids encoding fusions between the DNA-binding domain of LexA and various amino terminal regions of PDE4D5 were tested for their ability to interact with RACK1, expressed as a fusion with the GAL4 activation domain (left column "pGADN-RACK1"). The identical LexA fusions were tested for their ability to interact with the GAL4 activation domain alone (right column "pGADN"). The regions of PDE4D5 included in the various constructs are annotated. The positions of the portions of PDE4D5 used in this study are shown schematically in Figure 1. The interactions between these components were evaluated with the filter beta-galactosidase assay described previously by us [16,26]. The bottom two patches serve as internal positive (being the interaction between the oncoproteins RAS^{v12} and RAF) and negative (vectors with inserts) controls, respectively.

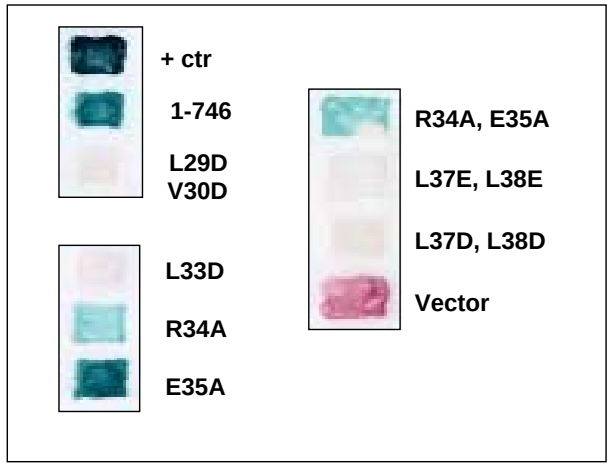

Figure 3
Identification of residues involved in the interaction between PDE4D5 and RACK1 using a 2-hybrid screen. Shows a typical experiment of one done at least 3 times, where mutations of individual or pairs of residues in the indicated N-terminal region of full length PDE4D5 were expressed as LexA fusions of the non-mutated PDE4D5 (1–746), pGADN-RACK1 and LexA alone ('vector'). The interaction between the oncoproteins RAS^{v12} and RAF served as a positive control

fect of individual mutations on the interaction. Each amino acid in this region was separately mutated to alanine except for the hydrophobic amino acids that were changed to aspartate and Ser-32 that was changed to both alanine and aspartate. This analysis highlighted a single residue, Leu-33, whose mutation to either aspartate (Fig. 3) or glutamate (not shown) ablated the interaction with RACK1. No other single mutations in the 28 through 49 region of PDE4D5 served to ablate interaction with RACK1 (data not shown), although we did observe a marked reduction in interaction with the Arg34Ala mutant (Fig. 3).

We noted, however, that the region C-terminal to the Asn-22/Pro-23/Trp-24/Asn-26 grouping contained a series of three leucine residues interposed by three amino acids in the repeat sequence Leu-29, X_{aa}, X_{aa}, X_{aa}, Leu-33, X_{aa}, X_{aa}, X_{aa}, Leu-37. This is reminiscent of hydrophobic repeat motifs that are found in amphipathic helical regions where coiled-coil forms of protein – protein interactions occur [40–43]. This repeat in PDE4D5 contains Leu-33, which we show here (Fig. 3) to be essential for PDE4D5 to interact with RACK1. As Leu-29 and Leu-37 both have hydrophobic residues immediately adjacent to them, namely Val-30 and Leu-38, we surmised that our inability to ablate interaction with RACK1 using single mutations might be due to contributions from these adjacent hydrophobic residues. We thus set out to evaluate whether mu-

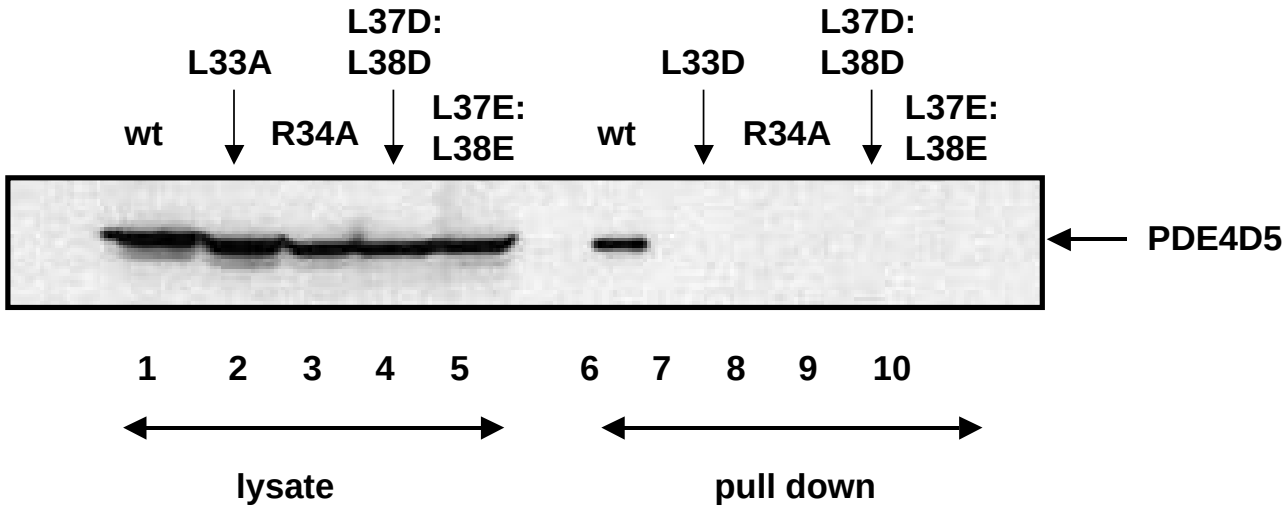

Figure 4
Pull down assays to probe the interaction between PDE4D5 and RACK1. Pull down assays between RACK1 and PDE4D5 mutants were done as described in Methods. Shown are immunoblots for VSV-epitope tagged forms of full length PDE4D5 detected using an anti-VSV monoclonal antibody. Tracks 1 to 5 are immunoblots of cell lysates showing that equal amounts of immunoreactive material were taken for these pull-down studies. Tracks 6 to 10 are blots showing the 'pull-downs', namely material that is found associated with glutathione agarose-immobilized GST-RACK1. Full length PDE4D5 forms were used as either wild type (tracks 1, 6) or the mutant forms Leu33Asp (tracks 2, 7), Arg34Ala (tracks 3, 8), Leu37Asp:Leu38Asp (tracks 4, 9) and Leu37Glu:Leu38Glu (tracks 5, 10). These data are typical of experiments done at least three times.

tation of these hydrophobic pairs would disrupt interaction with RACK1. Indeed the mutation of the Leu-29:Val-30 pair, to either aspartate (Fig. 3) or to glutamate (not shown), served to ablate interaction with RACK1. Similarly, mutation of the Leu-37:Leu38 pair, to either aspartate or to glutamate, also ablated interaction with RACK1 (Fig. 3). This suggests to us that these repeated hydrophobic residues are important in the binding of PDE4D5 to RACK1. We did note, however, that Leu-25, within the Asn-22/Pro-23/Trp-24/Asn-26 grouping, is also located three residues N-terminal to Leu-29 and thus falls within a repeating Leu, X_{aa}, X_{aa}, X_{aa}, Leu unit. However, previously we have shown that, unlike other residues within the Asn-22/Pro-23/Trp-24/Asn-26 grouping, the mutation of Leu-25 to alanine did not ablate binding to RACK1. In this study we additionally mutated Leu-25, to either aspartate or to tryptophan, with no ablation of RACK1 interaction as assessed by 2-hybrid analysis (data not shown). Thus Leu-25 does not appear to be essential for interaction with RACK1 in the way that either the surrounding residues in the Asn-22/Pro-23/Trp-24/Asn-26 grouping are or as Leu-33 has been shown to be (Fig. 3).

We also explored various other pairs of amino acids through their simultaneous mutation to alanine; predominantly to evaluate residues that might be involved in charge-charge or hydrogen bonding interactions. These were the pairs Arg-34/Glu-35, Gln-39/His-40, Glu-41/Lys-42, Ser-43/Thr-45 and Arg-47/Lys-48. Of these, only the double mutant, involving Arg-34/Glu-35, attenuated the interaction. This reduction in signal was clearly less than the ablated signal seen, for example, with the Leu33Asp mutation (Fig. 3). However, the apparent reduction in signal was comparable to that seen with the single Arg34Ala mutant (Fig. 3; right panel). In contrast to this, the single Glu35Ala mutant showed a similar signal to the control, wild-type PDE4D5 construct (Fig. 3). This suggests that Arg-34 and not Glu-35 plays a role in mediating the interaction between PDE4D5 and RACK1.

We wished to provide independent confirmation that residues in addition to those forming the Asn-22/Pro-23/Trp-24/Asn-26 grouping were important in the interaction between PDE4D5 and RACK1. To do this, the various Leu33Asp, Leu37Asp:Leu38Asp, Leu37Glu:Leu38Glu and Arg34Ala mutants were created in a VSV epitope-tagged version of PDE4D5 and transiently expressed in COS7 cells. Extracts from these cells were then subjected to a pull-down assay with GST-RACK1 as done before by us [26]. This analysis (Fig. 4) demonstrated that all of these mutations disrupted the interaction between PDE4D5 and RACK1. These data (Fig. 4) are consistent with the

two-hybrid results (Figs 2, 3) in highlighting an additional region of interaction within the unique N-terminal region of PDE4D5 that is essential for interaction with RACK1.

Whilst the 12–38 truncate of PDE4D5 contained both the Asn-22/Pro-23/Trp-24/Asn-26 grouping and the essential hydrophobic ridge, it failed to bind to RACK1 in the 2-hybrid screen (Fig. 2). It should be noted, however, that the final two residues of this truncated segment, namely Leu-37 and Leu-38, are together essential for allowing interaction with RACK1. It is thus highly likely that either steric influences or disruption of appropriate helix formation through replacement with 'foreign' residues, immediately C-terminal to Leu-37 and Leu-38 in the 2-hybrid construct, underpins this observation.

A 38-mer peptide, comprising residues 12–49 of PDE4D5, contains both the previously identified Asn-22/Pro-23/Trp-24/Asn-26 grouping [26] and also the novel leucine repeat unit shown also to be crucial for RACK1 to bind to PDE4D5. This peptide bound to RACK1 with similar affinity[1] (K_a of 6.4 ± 1.2 nM (Mean ± SD; n = 3 separate experiments) to that reported for PDE4D5 [39]. This is consistent with the 2-hybrid screen done in this study (Fig. 2), which indicated that the construct expressing amino acids 12 to 49 of PDE4D5 interacted with RACK1 in a similar fashion to full length PDE4D5. Thus the core PDE4D enzyme does not have any profound effect on the functional capability of the N-terminal region of PDE4D5 to interact with RACK1.

Molecular modeling

Molecular modeling studies (Fig. 5a), as well as the protein structure prediction programs, PHD and SSpro (data not shown) [44], suggest that the RACK1 Interacting Domain 1, RAID1 that we have identified here in PDE4D5 is likely to form a helical structure. In this the essential hydrophobic residues (Leu-29, Val-30, Leu-33, Leu-37, Leu-38) is suggested to provide a distinct hydrophobic ridge along one face of an amphipathic helix (Fig. 5a). This hydrophobic ridge (Fig. 5a) appears to comprise 3 key sticky 'patches', with 'patch 1' formed by Leu-29 and Val-30, 'patch 2' by Leu-33 and 'patch 3' by Leu-37 and Leu38. Destruction of any one of these patches in its entirety suffices to ablate interaction with RACK1. However, single mutations, to the charged amino acid aspartate, within either patch 1 or patch 3 are not sufficient to ablate interaction of PDE4D5 with RACK1.

At the N-terminal 'head' of this proposed hydrophobic ridge is found the Asn-22/Pro-23/Trp-24/Asn-26 grouping that is also essential for interaction of PDE4D5 with RACK1. Leu-25 (Fig. 5a) appears at first glance to form a simple continuation of this hydrophobic ridge into the

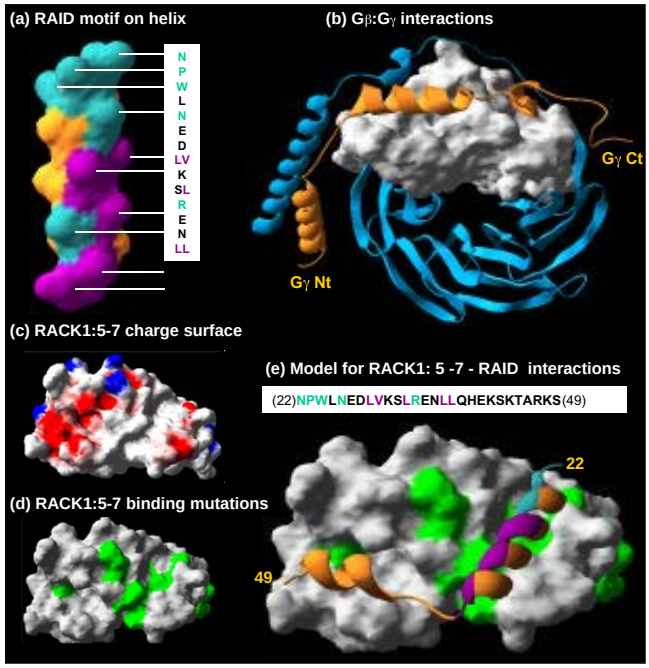

Figure 5
Modeling RAID-RACK1 interactions. (a) Surface representation of RAID1 core segment, Asn-22 to Leu-38, as a helix, with sidechains curtailed at the C_β atom for clarity. Residues implicated in RACK1 binding are colour-coded on a yellow helical background; blueAsn-22, Pro-23, Trp-24, Asn-26, Arg-34, and purple Leu-29, Val-30, Leu-33, Leu-37, Leu-38. These residues tend to align along one side of the helix. (b) G_β (blue) – G_γ (yellow) interactions from protein data bank coordinate set '1got,' with a molecular surface (white) drawn for the WD repeats 5–7 of G_β (lacking the initial β-strand of WD repeat 5 that is associated with the previous propeller blade). (c) Electrostatic potential surface of comparative modeled RACK1 WD repeats 5–7 (equivalent part and in the same orientation as that drawn for G_β in panel b). Red denotes negative, blue positive, and white is non-polar. (d) RACK1 mutations that affect RAID binding are drawn in green on a molecular surface for WD repeats 5–7. (e) Model for RACK1-RAID interactions, with the core RAID segment of panel a forming the right-hand helix, that connects through a loop to a positive region of RAID that is modeled (left-hand helix) into a negative part of the RACK1 surface. Colour coding for the RACK1 surface follows that in panel 2, and that for the core RAID segment follows panel a. Arg-34 is at the back of the helical ribbon and not visible in this view. The orientation matches panels b,c, and d, so that general features can be compared with G_β-G_γ.

Asn-22/Pro-23/Trp-24/Asn-26 grouping. However, its mutagenesis to a range of charged and aromatic amino acids did not affect the ability of PDE4D5 to interact with RACK1, suggesting that Leu-25 has little or no role in determining the interaction between these two proteins. Thus the Asn-22/Pro-23/Trp-24/Asn-26 grouping and the

proposed hydrophobic ridge that encompasses Leu-29, Val-30, Leu-33, Leu-37 and Leu-38 appear to form two discrete units within RAID1 that are each essential for PDE4D5 to bind to RACK1.

Discussion

We have defined here a sub-domain, which is located within the unique 88 amino acid N-terminal region of PDE4D5, that allows PDE4D5 to bind to the signaling scaffold protein, RACK1. This region was defined using independent methodologies of 2-hybrid assays and biochemical pull-down assays. We propose calling this region RAID1, for RACK1 Interacting Domain 1. RAID1 is located in the N-terminal half of this 88 amino acid isoform-specific region of PDE4D5, extending from Asn22 to Val30. It consists of a small cluster of amino acids, namely the Asn-22/Pro-23/Trp-24/Asn-26 grouping [26], together with a series of hydrophobic amino acids, namely Leu-29, Val-30, Leu-33, Leu-37 and Leu-38 in a 'Leu-X_{aa}-X_{aa}-X_{aa}-Leu' repeat.

The WD-repeat protein G_β, illustrated in Fig. 5b, interacts with the G_γ protein through coiled-coil interactions between the N-terminal regions of each subunit, and through helical and non-helical segments of the G_γ C-terminus, interacting largely with propeller blades 5–7 at the C-terminus of G_β[35,36,43,45]. Hydrophobic surfaces play a large role in these interactions. Using a truncation strategy coupled with a reverse 2-hybrid screen, we [46] have demonstrated that the C-terminal region of RACK1 plays a crucial role in conferring the binding of PDE4D5. Indeed, various single amino acid mutations in this region of RACK1 serve to ablate the binding of PDE4D5 [46]. Using colour coding of modeled RACK1 repeats 5–7 we identify here regions of particular polarity (Fig. 5c) as well as those that have been implicated through mutagenesis in binding PDE4D5 (Fig. 5d). Whilst RACK1 lacks an equivalent to the N-terminus of G_β, which mediates the helical coiled-coil interactions with G_γ, [45] these mutagenesis data and surface properties indicate that PDE4D5-RACK1 interactions may occur in a manner generally akin to that for the C-terminal regions of G_γ and G_β. We have attempted to explore this possibility in the model shown in Fig. 5e. The starting point for the model (Fig. 5e) is placement of RAID1, modeled as a helix (Fig. 5a), adjacent to the mutagenesis-implicated groove on RACK1 (Fig. 5d). Of the other major surface regions highlighted in Fig. 5d, that to the far right, by comparison with Fig. 5b, forms internal interactions and the upper central green feature is due to a single extended residue, R245, which would appear to be sufficiently flexible as to allow, in principle, an interaction with E35 in the PDE4D5 N-terminal region. Having placed the core segment of RAID1 in the model, we note that a second helical segment, containing a positively charged region of the PDE4D5 N-terminus, could be positioned to interact with a negatively charged region of RACK1 (Fig. 5c). Thus Fig. 5e shows both segments of the model, with a short connecting loop that mediates a change in helical direction. We note that in overall terms the model has similarities with G_β-G_γ binding [35,36,43,45], using helical segments binding into grooves and intervening turns. The reverse direction of the RAID1 polypeptide chain relative to G_γ, on the propeller surface, results from docking the positively charged RAID1 segment into a negatively charged part of the RACK1 model. Since the role of this part of RAID1 in binding is not clear, we regard this element of the modeling as secondary to the primary location of the amphipathic RAID1 helix in the RACK1 groove having the identified [46] multiple binding mutants. This primary docking could in fact be accomplished with either direction of polypeptide chain, so that we assign less weight to our suggestion of RAID1 running opposite to G_γ than to the proposed binding groove for the core RAID segment. Interestingly, Sondek and Siderovski ([47]), based on the established interaction between G_β and G_γ, used 3D-modeling analyses to propose that protein-protein interaction involving the C-terminal region of β-propeller proteins may be directed by putative G-protein Gamma-Like (GGL) motifs on the binding partner. Indeed, they went on to suggest that the Asn-22/Pro-23/Trp-24/Asn-26 grouping, in the unique N-terminus of PDE4D5, might resemble such a GGL domain and therefore bind to the C-terminal region of RACK1 in a similar region to that where G_β and G_γ have been shown to interact. Thus, by analogy with the GGL model ([47]), Asn-22/Pro-23/Trp-24/Asn-26 may represent a common core motif as seen with homologous proteins. If this is the case then the specificity of interaction must come from additional structural motifs that either enhance or reduce interaction with particular β-propeller proteins. On this basis, we suggest that the leucine-rich region, which we have identified in the present study, may serve primarily to direct specificity for interaction of RAID1 with RACK1 rather than other β-propeller proteins. The existence of this type of "structural conditioning" would mean that a particular family of β-propeller proteins could have different specificity with regards to their protein-binding partners. Additionally, for each β-propeller protein there may be a family of proteins that can even interact at one 'site'. From the 3D-models presented in this study (Fig. 5) we can see that WD 5–7 of RACK1 contains a range of different interaction sites and therefore may accommodate a range of possible interactors; including those that bind weakly by only interacting with a small part of the surface, and those that interact strongly, like RAID1 (Fig. 5), which interact with multiple determinants over an extended surface.

We also noted, however, that mutation, to alanine, of the positively charged residue, Arg-34 ablated PDE4D5 inter-

action with RACK1 in pull-down studies and severely attenuated interaction in 2-hybrid screens. This indicates that Arg-34 is also important in defining the binding of PDE4D5 to RACK1. Arg-34 is proposed as being located within the helix that contains the hydrophobic ridge and is adjacent to the negatively charged Glu-35. In our model it is possible that Arg-34 could either interact directly with RACK1, perhaps through Asp-294, adjacent in the model, or perhaps by virtue of non-polar interactions mediated by the aliphatic part of an arginine sidechain. Whilst, potentially, it could also stabilise a bound helical segment through interaction with Glu-35 we consider this to be unlikely as the Glu35Ala mutation interacts with RACK1 akin to wild-type PDE4D5.

Conclusions

Thus, an amphipathic helical conformation is suited to the part of PDE4D5 that we show here is essential for its binding to RACK1, namely Leu-29, Val-30, X_{aa}, X_{aa}, Leu-33, Arg-34, X_{aa}, X_{aa}, Leu-37, Leu-38. This helix may provide the driving force for an initial interaction between PDE4D5 and RACK1 that is then further stabilized through the correct presentation of the essential Asn-22/Pro-23/Trp-24/Asn-26 grouping. The structural model that we propose may describe such an interaction suggests that binding of PDE4D5 to RACK1 may involve interactions that are akin to those seen for the binding of the G_γ protein to G_β in terms of helix/groove nature and overall location. This interaction may thus allow PDE4D5 to be recruited into a signaling scaffold complex where RACK1 acts as an adaptor. Our definition of the interaction domain, RAID1 should aid in the design of reagents aimed at disrupting the interaction between PDE4D5 and RACK1 so as to use them to gain insight into the physiological function of such interaction in intact cells. Certainly, the targeting of PDE species is likely to contribute to the generation of compartmentalised cAMP responses [10]. Indeed, there is currently much interest in identifying anchor proteins for protein kinase A (AKAPs) that, seemingly, serve to sample gradient of cAMP within cells, leading to the selective activation of specific PKA isoforms that are associated with particular signalling functions [5,48,49].

Methods

Materials

An antibody to RACK1 was obtained from Transduction Laboratories. A monoclonal antibody to the VSV epitope tag [50] was from Sigma. The polyclonal antiserum specific for PDE4D was as described before by us [25]. Peptides were from Cambridge Biosciences (Cambridge, UK) and were determined to be >98% pure with composition verified by HPLC and mass spectrometry.

Two-hybrid constructs and assays

Various segments of the PDE4D5 cDNA (GenBank™ accession number AF012073) were cloned into the Not I site of the vector pLEXAN, to generate fusions between the various amino-terminal portions of PDE4D5 and the DNA binding domain of the *E. coli* LexA protein [26]. pLEXAN is a derivative of pBTM116, with a Not I site inserted into the polylinker. These constructs were prepared by the addition of Not I sites to the cDNA regions by the use of PCR, as described previously by us [26,51,52]. Similarly, the full open reading frame (ORF) of human RACK1 cDNA (ref. [28]; GenBank accession number M24194) was cloned into the Not I site of pGADN, to produce pGADNRACK1. pGADN is a derivative of pGADGH, but with a Not I site inserted into the polylinker. This vector expresses proteins as in-frame fusions with the trans-activation domain of the S. cerevisiae GAL4 protein. Two hybrid assays were performed in the *S. cerevisiae* strain L40 using methods that we have described in detail previously [16,26,52].

Generation of bacterial expression constructs

Various portions of the PDE4D5 cDNA were cloned into the Not I site of pMALN, using PCR as described above for the LexA fusions. pMALN is a derivative of pMALC2 (New England Biolabs), with a Not I site inserted into the polylinker. All PCR-generated or mutant constructs were verified by sequencing prior to use.

Generation of cDNAs encoding mutant forms of PDE4D5

To generate mutations of one or two individual amino acids in PDE4D5, the full-length PDE4D5 cDNA [25] was subjected to site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene). All PCR-generated or mutant constructs were verified by sequencing prior to use.

Expression of glutathione S-Transferase (GST) and maltose binding protein (MBP) fusion proteins in E. coli

The generation, expression and purification of fusion proteins between the full ORF of RACK1 and both glutathione-S-transferase (GST) and maltose-binding protein (MBP) have been described in detail previously by us [26].

RACK1 pull-down assays

The construction of pcDNAPDE4D5VSV has been described previously by us [26]. It contains the full ORF of PDE4D5, with a vesicular stomatitis virus (VSV) glycoprotein epitope at the carboxyl terminus [50], cloned into pcDNA3 (Invitrogen). Transfection of COS7 cells and pull-down assays were performed as described previously by us [25,26]. Briefly, COS7 cells were transfected with 20 μg of control plasmid pcDNA3 or with 20 μg of plasmid pcDNAPDE4D5VSV encoding wild type VSV-tagged PDE4D5, or with 20 μg of plasmids

pcDNAPDE4D5L33DVSV (Leu33Asp), pcDNAPDE4D5R34AVSV (Arg34Ala), pcDNAPDE4D5L3738DVSV (Leu37Asp:Leu38Asp), pcDNAPDE4D5L3738EVSV (Leu37Glu:Leu38Glu) encoding various mutant forms of VSV-tagged PDE4D5 (as indicated in parentheses). 72 h after transfection, cells were harvested in lysis buffer (55 mM Tris-HCl, pH 7.4, 132 mM NaCl, 22 mM sodium fluoride, 11 mM sodium pyrophosphate, 1.1 mM EDTA, 5.5 mM EGTA, 0.1% (v/v) Triton X-100 plus protease inhibitors) and incubated for a further 1 h with rotation at 4°C. Cell debris was removed by centrifugation at 13,000 *g* for 10 min at 4°C. 400 μg of cleared lysate was incubated with 50 μg of GST or GST-RACK1 and 60 μl of glutathione-Sepharose beads for 2 h at 4°C with rotation. Beads were pelleted by centrifugation at 2000 *g* for 5 min at 4°C, washed three times in lysis buffer and then boiled in 75 μl Laemmli buffer. 50 μl samples from beads and 50 μg each of cleared lysate were analysed by SDS-PAGE followed by immunoblotting with an anti-VSV monoclonal antibody [25,26].

SDS polyacrylamide gel electrophoresis and immunoblotting

These were as described previously [25,26]. In brief, samples were resuspended in Laemmli buffer and boiled for 5 min. Membranes were blocked in 5 % (w/v) low-fat milk powder in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at room temperature. They were then incubated with anti-VSV monoclonal antibody diluted in 1:5000 (v/v) in 1 % (v/v) low-fat milk powder in TTBS (TBS plus 0.1 % (v/v) Tween 20) for 3 h at room temperature. Detection of the bound antibody was with anti-mouse IgG peroxidase (Sigma) and the enhanced chemiluminescence (ECL) system (Amersham).

Measurement of protein concentrations

Protein concentrations were measured by the method of Bradford [53], using BSA as a standard.

ELISA binding assay

A modification of the method described previously by us [26] was used. High binding ELISA plates were treated with 100 μl of MBP-PDE4D5, MBP alone or various peptides at a concentration of 1 μM in 50 mM carbonate-bicarbonate buffer (Sigma C-3041) for 16 h at 4°C. The plates were then blocked in 5% (w/v) low-fat milk powder in above buffer for 1 h at room temperature. Plates were washed three times with 200 μl/well wash buffer (TBS + 0.1% (v/v) Tween-20) before being incubated for 2 h, at room temperature, with 100 μl GST-RACK1 solution in TBS. The GST-RACK1 solution was formed from a range of dilutions over the concentration range 50 pM – 2 μM. Plates were then washed as above with protein complex formation being detected by the addition of anti-RACK1 monoclonal antibody followed by peroxidase conjugated anti-mouse IgM. These antibodies were both used at 1:2500 in TBS for 1 h at room temperature with a wash step in between. Immunoreactivity was visualized by adding ABTS solution (0.4 mM 2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] in 50 mM sodium citrate pH 4.0 plus 36 μl 30% (vol/vol) H_2O_2 just prior to use) and quantified using an MRX plate reader set at a wavelength of 405 nm.

Molecular modeling

A comparative model of RACK1 was constructed with the program Modeller [54], using bovine transducin G_β as the structural template. The G_β subunit was extracted from a G protein heterotrimer structure [45] with coordinate file identifier 1 got in the protein structural data bank. The underlying sequence alignment between RACK1 and the G_β template followed that given previously [46], except for WD repeats 6 and 7 where the sequence match is less clear and structural factors were used to make small adjustments. In particular, putative β-strands in RACK1 were identified through alignment of stretches of non-polar residues with the known β-strands of the G_β subunit, which are also predominantly non-polar. This adjustment aligned W339, N340 of G_β with W310, Q311 of RACK1. Analysis of the RACK1 comparative model in terms of binding the N-terminal region of PDE4D5 focussed on WD repeats 5–7. Molecular surfaces were drawn for WD repeats 5–7 that lack the start of WD repeat 5, since this region would form the outer β-strand of an otherwise absent propeller 4, and is therefore unlikely to exhibit structural order in the experimental WD repeat 5–7 construct. The N-terminal region of PDE4D5 implicated in binding to RACK1 by mutational analysis was modelled as mainly α-helical polypeptide, with the potential for non-helical linker regions. Binding surfaces on RACK1 and PDE4D5 were assigned from the mutation affects on binding, and their combination in a general model for complexation used these data as well as overall complementarity of surface shape and polarity. Computer programs QUANTA (Accelrys) and Swiss-PDB Viewer [55] were used for manipulation and display.

Authors' contributions

MDH and GBB conceived, directed and managed the study. GBB and MRS performed the 2-hybrid analyses. AM performed the biochemical pull-down studies and had input into the direction of the study. SJW performed the ELISA studies and had input into the direction of the study. JW performed the molecular modeling analyses.

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